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Review of Ammonium Dinitramide Toxicity Studies

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14. ABSTRACT
Ammonium dinitramide (ADN) is a class 1.1 explosive oxidizer being considered for use in solid rocket propellant mixtures. Studies performed evaluating the toxicity of ADN have included an acute and subacute toxicity screen, a 90-day reproductive screen and three follow-on reproductive studies, electron paramagnetic resonance (EPR) spectroscopy studies, mutagenicity assays and a study evaluating the effects of ADN on hepatocytes in vitro. The LD_{50} in rats is 823 mg/kg, indicating ADN is moderately toxic. The results also indicated that ADN is a female reproductive toxicant in rats, causing implantation failure in early gestation; follow-on studies implied that ADN is embryotoxic. EPR studies indicated that ADN can decompose to form reactive nitrogen metabolites which can be harmful in biological systems. The hepatocyte studies suggested that ADN has the potential for directly affecting cellular DNA in vitro; these results are supported by traditional genotoxicity assays which reported that ADN is mutagenic.

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PREFACE

This review was conducted under the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. (HJF) contract, FA8650-05-2-6518. The program manager for the contract was Mark Hoffman of the Air Force Research Laboratory, 711 Human Performance Wing, Human Effectiveness Directorate, Biosciences and Performance Division (711 HPW/RHP). The technical manager for the project was David Mattie, Ph.D. in the Applied Biotechnology Branch of 711 HPW/RHP.

The animal studies were approved by the Air Force Surgeon General's Research Human & Animal Research Panel and the Wright-Patterson Air Force Base Institutional Animal Care and Use Committee. The studies were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996).

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1.0 EXECUTIVE SUMMARY

Ammonium dinitramide (ADN) is a class 1.1 explosive oxidizer, which has been repeatedly evaluated for use in solid rocket propellant mixtures. A number of studies were performed evaluating the toxicity of ADN, but a review of its toxicity has never been published. Toxicity studies have included an acute and subacute toxicity screen, a 90-day reproductive screen and three follow-on reproductive studies, electron paramagnetic resonance (EPR) spectroscopy studies, mutagenicity assays and a study evaluating the effects of ADN on hepatocytes *in vitro*. The LD_{50} in rats is 823 mg/kg, indicating that ADN is moderately toxic. No evidence of dermal irritation or general toxicity was found. ADN is a female reproductive toxicant in rats, causing implantation failure in early gestation. The follow-on reproductive studies, reproduction and fertility, pre-implantation and post-implantation studies, implied that ADN is embryotoxic. A mouse embryo toxicity study also showed ADN affects the embryo. The *in vitro Hydra attenuata* developmental toxicity screen did not support the rodent developmental toxicity data. EPR studies indicated that ADN can decompose to form reactive nitrogen metabolites which can be harmful in biological systems. The hepatocyte studies suggested that ADN has the potential for directly affecting cellular DNA *in vitro*. The EPR and hepatocyte results are supported by traditional genotoxicity assays, including the Ames test, the mouse lymphoma cell mutagenesis test, and the *in vivo* mouse bone marrow micronuclei assay, which all reported that ADN is mutagenic.

2.0 INTRODUCTION

Ammonium dinitramide (ADN) is a class 1.1 explosive oxidizer which has been considered for use by DoD and NASA in solid rocket engine propellant mixtures and explosives (Kinkead *et al.*, 1995). ADN is a potential replacement for ammonium perchlorate (AP) in rocket propellant formulations. ADN is predicted to provide better performance (between 5 and 15 percent) and a potential 8 percent payload increase (Borman, 1994). Burning AP produces a heavy smoke trail with high hydrochloric acid content. Chlorine is a major contributor to ozone depletion in the stratosphere and the highly visible contrail makes rockets more vulnerable to detection and tracking (Berty *et al.*, 1995). ADN should not generate a heavy contrail. In 1990, the Armstrong Laboratory, now Air Force Research Laboratory, was tasked by the USAF with assessing the occupational and environmental risk of ADN (Steel-Goodwin *et al.*, 1995a). Recent re-interest in ADN as a propellant revealed that its toxicity in the occupational setting had never been summarized in accessible literature.

ADN ($NH_4N(NO_2)_2$) is a light sensitive, white, water soluble powder which must be stored in protective vials in an enclosed cabinet. The test compound, as commonly available through SRI International (Menlo Park, CA), is known to be contaminated with 1 to 2 percent ammonium nitrate (Graeter *et al.*, 1998). ADN in aqueous solution is stable in the absence of ultraviolet light. In the presence of UV light, ADN decomposes, accompanied by a decrease in solution pH (Kinkead *et al.*, 1995; Steel-Goodwin *et al.*, 1997). Breakdown products in water include the free radical NO^-, as well as NO_2 and NO_3 (Steel-Goodwin *et al.*, 1997).

3.0 TOXICITY STUDIES

Prior to the studies initiated by Armstrong Laboratory in 1990, no toxicological information was available for this compound. Anecdotal field reports of ADN causing numbness of the fingers indicated that the oxidizer is readily absorbed into the skin (Kinkead *et al.*, 1995). The routes of exposure considered most viable were dermal absorption and accidental ingestion of ADN. Environmental exposure through drinking water contamination also constitutes a potential exposure route. Since ADN is not volatile (calculated vapor pressure of 1.7×10^{-12} at 25°C) (Clausen *et al.*, 2007), inhalation exposure to the oxidizer would be minimal.

3.1 Acute Toxicity

ADN was tested in standard acute studies. The oral LD_{50} in young adult male F-344 rats was determined to be 823 mg/kg (Table 1); the mean time to death was less than one hour. This result classifies ADN as "moderately toxic." All deaths were preceded by convulsions; systemic vasodilation typically caused by nitrates was also evident (Kinkead *et al.*, 1994).

No evidence of dermal irritation or toxicity was found when ADN was tested in male New Zealand white rabbits, the most common model for human dermal irritation. ADN was applied at a dose of 2 g/kg topically to the backs of the rabbits (approximately 10 percent of the rabbit's

surface area) (Table 1). The application site was occluded for 24 hours and the rabbits were monitored for 14 days post-exposure (Kinkead *et al.*, 1994).

Table 1. Results of Standard Acute Toxicity Tests with ADN

Test	Species	Concentration	Result
Oral LD_{50}	Male F-344 rat	823 mg/kg	Moderately toxic
Dermal irritation & toxicity	Male NZW rabbit	2 g/kg	No evidence of irritation or toxicity

Note: All results from Kinkead *et al.* (1994); F-344 = Fischer 344; LD_{50} – 50% lethal dose; NZW = New Zealand White

In addition, a three-week palatability study was conducted to assess whether ADN drinking water studies were feasible. Young adult, male and female Sprague-Dawley (SD) rats consumed a range of 6 to 120 mg ADN/kg/day without clinical signs of stress (Table 2). Water consumption rates were not different than control rates. There were no indications of toxicity during the standard necropsy (Kinkead *et al.*, 1994).

Table 2. Results of Repeated Dose Drinking Water Studies with ADN

Test	Species	Duration	Concentration (mg/kg/day)	Result
Drinking Water Palatability	M & F SD rat	21 days	6 – 120	No indication of toxicity[1]
SIDS: General Toxicity Parameters	M & F SD rat	90 days	M: 0, 17, 88, 146 F: 0, 29, 103, 162	No effect on body or organ weight No effect on hematology, clinical chemistry, histopathology[2]

Notes: [1]Kinkead *et al.* (1994); [2]Kinkead *et al.* (1995); F = female; M = male; SD = Sprague-Dawley

3.2 Repeated Exposure Study

A Screening Information Data Set (SIDS) test provides general toxicity, reproductive toxicity, and developmental toxicity information following repeated administration of the test material. Male and female SD rats were exposed through drinking water for 90 days. Consumption of the drinking water, which was decreased in the high dose animals, resulted in mean doses of 0, 17,

88 or 146 mg ADN/kg daily in males. Average doses for females were 0, 29, 103 or 162 mg/kg daily. Body weights, organ weights and relative organ weights were not affected by exposure to ADN (Table 2). Routine hematology, clinical chemistry and histopathology were normal following 90 days of exposure (Kinkead *et al.*, 1995).

3.3 Reproductive Studies

The SIDS test also measured reproductive and developmental endpoints. Sperm concentration, motility and morphology were not different from controls. A dose related decrease in number of litters was seen. Of the control dams, only 82 percent produced litters; 92, 25 and 10 percent of the dams produced litters in the 29, 103 and 162 mg/kg dose groups, respectively. The decrease in litters produced was significantly different from the control group in the mid- and high-dose female rats. Length of gestation, sex ratio of pups and pup weight for 21 days following parturition were not affected (Table 3). Histopathology did not identify a cause for decreased numbers of litters among ADN treated rats. The no observable adverse effect level was 29 mg/kg daily (Kinkead *et al.*, 1995).

Additional studies were performed to determine the mechanism of the reproductive effects of ADN. In the first follow-up study, female Sprague-Dawley rats were treated with ADN in drinking water, resulting in doses of 0, 26, 116 or 186 mg ADN/kg bodyweight daily. Females were exposed from 14 days prior to mating through gestation day (GD) 10 or GD 20. The number of corpora lutea did not differ between groups, implying that ovarian function was not impaired (Table 3). However, the number of fetuses at necropsy on GD 10 or 20 was significantly decreased in the mid- and high-dose groups. Again, the no observable adverse effect level was 26 mg/kg daily (Kinkead *et al.*, 1996).

In the second follow-up study, mated female SD rats received ADN at 211 or 199 mg/kg daily on GDs 1-3 or GDs 4-8, the pre-implantation or post-implantation periods, respectively. The pre-implantation group was found to have no implantations at all, while no effect was seen in the post-implantation group; both groups were necropsied on GD 9 (Table 3). The number of corpora lutea did not differ between groups. Serum progesterone, prolactin and progesterone were significantly decreased by ADN in both groups. These hormone levels are also driven by maternal signals and normal implantation/embryonic development, leaving the cause of implantation failure uncertain (Kinkead *et al.*, 1996).

In a third study, female SD rats were treated with drinking water containing approximately 200 mg ADN/kg/day from GD 0 for 24, 48, 72 or 96 hours. Embryos were collected from the oviducts and uterine horns and their number, location and stage of development were recorded. Embryo development in treated dams slowed or stopped from 48 through 72 hours, indicating that embryo lethality is at least partially to blame for preimplantation failure caused by ADN (Table 3) (Graeter *et al.*, 1998).

Two-cell mouse embryos were harvested from B6C3F1 females for the fourth study. Embryos were isolated 36 hours post-mating and cultured in medium containing 0, 1, 4, 6, 10, or 20 nM ADN. Development of the embryos was monitored via phase contrast microscopy at 24, 48 and

72 hours. Embryo development lagged at all concentrations of ADN in a dose-dependent manner; embryos in 1 nM ADN lagged 24 hours behind control embryos (Table 3). Degeneration of the embryos was seen after 72 hours in 4 nM ADN and higher, again in a dose- and time-dependent manner (Graeter *et al.*, 1996).

ADN was tested in an *in vitro* developmental toxicity screen with *Hydra attenuata*. Hydra are coelenterate invertebrates composed of tissues and organs, but with the ability for whole-body regeneration. In order to regenerate, hydra must achieve nearly all developmental events required of embryogenesis. Adult hydra and artificial embryos, pellets made of dissociated hydra cells, were incubated in ADN at concentrations of 100 to 1000 mg/L for 90 hours. An average of 750 mg ADN/L was lethal to adult hydra, while 350 mg/L was lethal to regenerating hydra (Table 3). ADN was not considered a primary developmental toxicant in the terms of this assay (Wolfe *et al.*, 1996).

Table 3. Results of Reproductive and Developmental Studies with ADN

Test	Species	Duration	Concentration	Result (*Dose Dependent)
SIDS: Reproductive / Developmental Parameters	Male SD rat	90 days, from 14 days prior to mating	M: 0, 17, 88, 146 mg/kg/day	No effect on sperm concentration, motility or morphology[1]
SIDS: Reproductive / Developmental Parameters	Female SD rat	90 days, from 14 days prior to mating	F: 0, 29, 103, 162 mg/kg/day	Decreased number of litters* & pups/litter No effect on length of gestation, sex ratio, pup weight NOAEL: 29 mg/kg/day[1]
Female Reproduction Study	Female SD rat	14 days prior to mating through GD 10 or GD 20	0, 26, 116 or 186 mg/kg/day	Decreased number of fetuses* No effect on number of corpora lutea NOAEL: 26 mg/kg/day[2]
Implantation Study	Female SD rat	Pre-implantation GD 1-3	211 mg/kg/day	No implantations No effect on number of corpora lutea Decreased serum progesterone, prolactin & estradiol[2]
Implantation Study	Female SD rat	Post-implantation GD 4-8	199 mg/kg/day	No effect on implantations No effect on number of corpora lutea Decreased serum progesterone, prolactin & estradiol[2]
Embryo Development Study	Female SD rat	GD 0 through 24, 48, 72 or 96 hours	~200 mg/kg/day	Slowed or stopped development of embryos at 48 – 72 hours Embryo lethality confirmed[3]
Mouse Embryo Study	Embryos (36 hours old) from B6C3F1 mice	*In vitro*, 24, 48 or 72 hours	0, 1, 4, 6, 10, or 20 nM	Slowed embryo development* Embryo degeneration at 72 hours (4 nM and higher concentrations)[4*]
Hydra attenuatta Developmental Toxicity Screen	Adult hydra & artificial embryos	*In vitro*, 90 hours	100 to 1000 mg/L	750 mg/L: Adult toxicity 350 mg/L: Developmental toxicity Not a primary developmental toxicant in the terms of this assay[5]

Notes: [1]Kinkead *et al.* (1995); [2]Kinkead *et al.* (1996); [3]Graeter *et al.* (1998); [4]Graeter *et al.* (1996); [5]Wolfe *et al.* (1996); F = female; GD = gestation day; M = male; NOAEL = no observable adverse effect level; SD = Sprague-Dawley

3.4 Genotoxicity Studies

Genotoxicity testing is routinely performed as one phase of the general toxicity screen of new compounds. The genotoxicity, or mutagenicity, of a compound refers to its ability to induce DNA damage and genetic alterations in cells. Such damage to germ cells can lead to increased genetic disease in offspring; unrepaired damage to somatic cells may lead to a carcinogenic event (Preston and Hoffmann, 2001). ADN was tested in three short-term genotoxicity assays: the Ames Test, the mouse lymphoma cell mutagenesis test, and the *in vivo* mouse bone marrow micronuclei assay.

The Ames *Salmonella*/mammalian microsome reverse mutation assay measures reversion from histidine dependent (his-) to histidine independence (his+) in *Salmonella* bacteria. Reversion is induced by base changes or frameshift mutations. *Salmonella* were exposed to ADN (dose range = 0.3125 – 5.0 mg/plate) both with and without metabolic activation (Aroclor-1254 induced rat liver S9 microsomes that simulate mammalian metabolic activation). ADN significantly increased mutations in *Salmonella* bacteria at a dose of 5 mg/plate (Table 4). Mutations were increased two-fold more than background reversion without S9 activation and three-fold with S9 activation in one *Salmonella* strain (TA100). No significant increases in mutation were seen in *Salmonella* strains TA1535, TA1537 and TA98 (Zhu *et al.*, 1994).

The mouse lymphoma cell mutagenesis (L5178Y-TK) test detects mutations at the thymidine kinase (TK) locus in mammalian cell culture. Cells were incubated in the presence of ADN (dose range: 0.05 – 5.0 mg/mL) and then switched to media containing trifluorothymidine (TFT). Cells without mutations to TK metabolize TFT to a cytotoxic metabolite. Cells without TK capability, due to forward mutations, survive in media containing TFT. ADN significantly increased mutations at the TK locus at a dose of 5.0 mg/mL (Table 4). TFT resistant mutants increased 40-95 percent (without S9 activation) or 130-220 percent (with S9 activation) over the control cell cultures (Zhu *et al.*, 1994).

The *in vivo* mouse bone marrow micronucleus (MN) assay detects damage of the chromosome or mitotic mechanisms. Male and female Swiss CD-1 mice were exposed to ADN by oral gavage at doses ranging from 62.5 to 750 mg/kg daily for 3 days. Polychromatic erythrocytes (PCE) were harvested from the bone marrow and micronuclei were examined. Micronuclei are small particles containing chromosomes or chromosome fragments that are formed when they lag behind during the anaphase of cell division. ADN increased micronucleated cells in a dose-dependent manner, with the highest doses increasing micronuclei by three-fold as compared to untreated controls (Table 4). Bone marrow toxicity (determined as a decrease in PCE/normochromatic erythrocytes ratio) was also observed in a dose-dependent pattern (Zhu *et al.*, 1994).

Table 4. Results of Genotoxicity Studies with ADN

Assay	Species, Concentration	Result
Ames Reverse Mutation	*Salmonella typhimurium* strain TA100, 5 mg/plate	Significant increase in mutations with S9 metabolic activation (3x background) or without activation (2x background)
Ames Reverse Mutation	*Salmonella typhimurium* strains TA1535, TA1537, TA98, 5 mg/plate	No effect on mutation as compared to background
L5178Y-TK	Mouse lymphoma cell, 5 mg/mL	Significant increase in mutations with S9 metabolic activation (130-220% above background) or without activation (40-95% above background)
In Vivo Bone Marrow Micronucleus	M & F Swiss CD-1 mice, 62.5 – 750 mg/kg/day, oral gavage, 3 days	Significant increase in micronucleated cells in dose-dependent manner 750 mg/kg/day: Increased micronuclei 3x control level Significant increase in bone marrow toxicity in dose dependent manner

Note: All results from Zhu *et al.* (1994)

Additional assays supported genotoxic findings. Enzyme leakage assays were performed to determine the levels of ADN that affected membrane integrity in WB344 hepatocytes and to quantitate the EC_{50} (the concentration of ADN *in vitro* that damages 50 percent of the exposed cells). The results of these assays indicated that ADN concentrations greater than 2.7 mM affected the membranes of 50 percent of the cells *in vitro* (Steel-Goodwin *et al.*, 1996). Data from the Yeast Del assay (Xenometrics, Inc., Boulder, CO) suggested that ADN can affect cellular DNA at concentrations below cytotoxic levels. Results from the stress gene induction assay suggest that ADN may act through an oxidative challenge mechanism in causing damage to nuclear DNA (Dean and Channel, 1995). Researchers using high pressure liquid chromatography (HPLC) and spin trapping found that ADN fragments deoxyribose nucleic acid (DNA) when incubated together *in vitro*. In the presence of oxygen, the number of free radicals detected in DNA isolates increased 828 percent (Steel-Goodwin *et al.*, 1997).

3.5 Potential Mechanism

Free radical mediated tissue injury is a well documented phenomenon. Free radical formation is thought to play a role in a growing number of disorders involving various organs and tissues in the body: the liver, brain, lungs, gastrointestinal tract, skin, kidney, and others (Kehrer, 1993).

Both oxygen and nitrogen centered free radicals have been implicated in the disease processes. Damage at the cellular level has been detected in biological molecules and structures including DNA, proteins, lipids, and membranes (Grisham, 1992). Damage to any of these cellular components may lead to alterations in cell viability or function. Free radicals do occur naturally in biological systems and cells have developed numerous defense mechanisms, including catalases, superoxide dismutases, the glutathione system, vitamin E, and ascorbate that prevent or repair free radical mediated damage (Kehrer, 1993). If these defense mechanisms are unable to keep pace with free radical formation, cellular damage ensues. This can be the case following exposure to exogenous compounds that decompose to form free radicals. The chemical structure of ADN indicates that its decomposition may yield nitrogen-centered free radicals.

Gamma-irradiation of ADN produced an electron paramagnetic resonance (EPR) spectrum showing two radical species. One species possesses a spectrum similar to the NH_3 radical, while the second species yielded a spectrum attributed to the NO_2 free radical. The second radical was shown to persist for over 20 hours (Steel-Goodwin *et al.*, 1995a). Electron-nuclear double resonance (ENDOR) experiments were also performed that corroborated these findings (Steel-Goodwin *et al.*, 1995b).

4.0 DISCUSSION

ADN is a female reproductive toxicant in rodents. The preliminary data indicate that ADN is toxic to preimplantation embryos *in vivo* and *in vitro*, leading to infertility. The mechanism by which the embryotoxicity occurs has not been identified. One study suggests that ADN disrupts the female endocrine system during pregnancy, although these data are somewhat ambiguous.

Traditional genotoxicity tests have shown that ADN is mutagenic to bacteria and mammalian cells *in vivo* and *in vitro.* Data from the Yeast DEL Stress Gene Induction assays suggest that ADN directly or indirectly damages nuclear DNA via an oxidative mechanism. Additional studies are required to confirm this hypothesis. These results indicate a strong potential for ADN to be mutagenic in humans (Mortelmans and Zeiger, 2000).

The decomposition of ADN generates at least two species of free radicals, NH_3 and NO_2, the second of which persisted for a twenty hour period *in vitro*. Either of these free radicals can be damaging to biological systems by attacking DNA, RNA, phospholipids, proteins and other cellular and biological molecules. Again, ADN may be acting through an oxidative mechanism; exposure to ADN may alter the levels of reactive oxygen and nitrogen species in biological systems causing oxidative stress.

5.0 CONCLUSIONS

Given the positive findings reported here, occupational exposure to ADN may pose a risk to human health. In reviewing the data, it is important to note that even low level exposure to ADN over time may adversely affect an individual and that the effects of exposure may not be immediately apparent. Additional studies that address the mechanisms of action of the effects of ADN reported here would prove very useful to the risk assessment community.

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LIST OF ABBREVIATIONS

ADN	ammonium dinitramide
AP	ammonium perchlorate
DNA	deoxyribose nucleic acid
EC_{50}	50% effective concentration
ENDOR	electron-nuclear double resonance
EPR	electron paramagnetic resonance
GD	gestation day
his	histidine
HPLC	high pressure liquid chromatography
MN	micronucleus
PCE	polychromatic erythrocytes
SD	Sprague-Dawley
SIDS	Screening Information Data Set
TFT	trifluorothymidine
TK	thymidine kinase